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The Genetic Diversity of *Aquilaria microcarpa* interacting with *Fusarium sp*

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Abstract

Aquilaria microcarpa is one of the agarwood-producing plants, which is currently very limited in nature. Plant defense systems are strongly influenced by the resistance genes possessed by plants. The genetic diversity of these plants would be seen by microsatellite markers as consideration in the development of this species. This research was conducted by observing the differences in gene characteristics from plants that were inoculated with Fusarium (n=40) and not inoculated (n=40). The results showed that of the 4 specific primers tested, only two primers were well amplified, with an average of 4 alleles per locus. The Polymorphism value of Information Content (PIC) at 6PA 18 locus in plant populations inoculated was 0.534. The value of genetic differential obtained from the two observed loci is high, indicating that the genetic diversity that occurs was not only influenced by the genetic makeup of plants, but also environmental factors where the plant grows.

Keywords: Aquilaria microcarpa, agarwood, Genetic diversity, microsatellite

1. INTRODUCTION

Aquilaria microcarpa is one of the plant species of the Thymeleacea family that is able to produce agarwood when interacting with *Fusarium sp* [1]. The agarwood product produced by this plant has a sales value of up to 30 million per kilogram, especially for super quality. The better the quality of the agarwood of a plant, the higher the value of the benefits of this plant, such as raw materials for perfumes and other beauty products. As such, the search for agarwood-producing plants in nature is getting higher which has an impact on the decline in the potential of these plants in nature. [2] states that *Aquilaria microcarpa* is one type of the genus *Aquilaria* which has been categorized as endangered (EN) under IUCN Redlist, a species whose numbers is at risk of extinction.

Agarwood is a form of accumulation in plant tissue against biotic and abiotic disorders. One of the causes of this biotic disorder is caused by the fungus *Fusarium* sp which plays an important role in the process of aloes formation [3];[4]. The response of plants posed is part of the physical and biochemical barriers carried out by plant tissues [5]. The defense mechanism carries out by plants vary greatly depending on plant varieties and a number of defense genes contained in individual plants [6]. The resistance of these genes will generally produce certain proteins, namely enzyme proteins or structural proteins that play a significant role in cellular and biochemical reactions in producing certain morphological properties or the accumulation of certain chemicals. This variation has an impact on plant genetic diversity.

Genetic diversity can be seen by using allele characters from a particular locus derived from plant tissue. This study aims to see the level of diversity between *A. microrocarpa* plants that have been inoculated and plants that have not been inoculated. This genetic characteristic is carried out with molecular markers because it has a high level of accuracy and efficiency. Microsatellite markers are molecular markers that have codominant properties with high polymorphisms [7][8]. This marker is also a very accurate tool to well distinguish plant genotypes. Based on these considerations, the level of plant genetic diversity is expected to be known, as a basis for information for plant breeding.

2. METHODS

Plant material used was the leaves of *A. microcarpa* obtained from KHDTK Carita Banten. Genetic analysis was conducted in Silviculture of forestry laboratories, Bogor Agricultural University. The method used for DNA extraction was the modification of CTAB (Cetyl Trimethyl Ammonium Bromide) method [9]. Most of the methods for extracting DNA from plant tissue contained in the literature, required a long time and expensive chemicals such as chromium chloride so that it is less efficient [10].

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The primers used in the study consisted of four specific primers for the *Aquilaria* type [11]. The primer was isolated and designed by Eurlings of the type *Aquilaria. crassna*. The four primers were 6pa18, 10pa17, 16pa17, and 71pa17 (Table 1).

Table 1 Primary microsatellite *Aquilaria crassna* according to Eurlings et al. (2010)

Locus	***Primer sequence* (5'–3')**	***Repeat***	***Size range* (bp)**	**Number of alleles**	**TA (^{O}C)**
6pa18	F: TGAGGCGTGAGTGAGATATTGATT R: CCTTCCTCTCTTCTTACCTCACCA	$(CA)_8$	180–210	7	50
10pa17	F: ACACACTGTTATGGTCTACAGCTT R: CGCCATCTCATAATATTCTAATGTA	$(CA)_{12}$	152–156	3	50
16pa17	F: AGTGAACAACTTGACTAGGCTTG R: GCTGAACACAACAAGATATCACC	$(CA)_{19}$	143–155	6	59
71pa17	F: AGCAAACAGTGGGATAAGGTC R: AGAAAGGAGGCGAAACGAAT	$(CA)_{15}$	152–224	15	54

Polymorphism testing was done by observing the results of the PCR tape visualized based on the results of electrophoresis. The results of this test were said to be polymorphic if the resulting tape pattern has at least more than one variation, while the test results were said to be monomorphic if they did not show any variation on the band pattern of electrophoresis.

Molecular data analysis was performed based on the results of scoring DNA bands that appeared on polyacrylamide gels, where at each locus was assumed to be a microsatellite allele. Data were analysed using Popgene 1.32 software programs, version of NTsys 1.80, GSDE and the AMOVA Table based on Arlequin version 3.1.

3. DISCUSSION

SSR Primary Amplification Capability

The results of DNA amplification testing using four pairs of SSR primers were migrated using electrophoresis in *A. microcarpa* populations. There were only two primers capable of amplifying DNA well (Figure 1). Two other pairs of primers namely 10 PA 17 and 16 PA 17, did not produce amplification products. [11] tested 12 microsatellite primers on *Aquilaria crassna* plant, but only 4 primers were able to amplify samples with polymorphic patterns, while one primer was monomorphic amplified and 7 other primers were not amplified. This is thought to be caused by incomplete PCR component completeness and conditions during the PCR process. The primary annealing condition is regulated by touchdown decrease which gives a variety of temperature ranges for the primary attachment to occur, so that the amplification failure due to temperature mismatch has been minimized. Based on this, the failure of amplification in some primers was not due to mismatch conditions during the PCR process. The failure of amplification in this study was possible because of the incompatibility of the primary sequence with the printed DNA sequence, as reported by [11] where from several *Aquilaria crasna* samples for different locations, the primary pair he designed could not amplify it. Primers used in this study have reported success in amplifying the DNA of agarwood-producing plants from various regions [12].

The 6 PA 18 and 71 PA 17 microsatellite primer pairs tested against *Aquilaria microcarpa* were able to amplify 5 to 6 alleles. These alleles are polymorphic (Table 1). A gene locus was to be polymorphic if there were at least two different allele variations and the frequency of the allele was often found to be less than 95% [13]. The size and number of alleles were carried out according to [14] assuming that all DNA bands with the same migration rate, were assumed to be homologous loci. The DNA profile data were then translated into distance matrix data based on heterozygosity values of the population being compared. The results of this microsatellite primer amplification can be used as a marker candidate in plants. However, to ensure the accuracy as the stability of the alleles produced, the use of 6 PA 18 and 71 PA 17, microsatellite markers needs to be carried out further or repeated tests, for example with a larger sample size and population and broad geographical coverage so that evaluation estimates would be used genetic or phylogenetic which can be determined based on this marker.

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Table 2. Data on the amplification capabilities of microsatellite primers in *A. microcarpa*

Primer name	Fragment Size (bp)	Amplification Ability	Fragment Length (bp)	Number of alleles	Information
6 PA 18	180-210	+	185,195,199,210,223	5	Polimorphic
10 PA 17	152-156	-	-	-	-
16 PA 17	143-155	-	-	-	-
71 PA 17	152-224	+	158,166,178,195,199,210,223	6	Polimorphic

Note: +: amplified, -: not amplified

Spread of microsatellite alleles

Nine microsatellite alleles were detected in three *A. microcarpa* population observed, with varying amounts of both 6 Pa 18 and 71 Pa 17 loci, with an average of 4 alleles per locus. The frequency of alleles for each locus of the inoculation and non-inoculation population tested (Table 3). [11] detected 3 to 15 alleles of the four polymorphic loci used in *Aquilaria crassna*.

Table 3 Frequency of alleles per locus in each population

Population	Number of samples (n)	Locus 6PA18						Locus 71PA17						
		180	185	195	199	210	223	158	166	178	195	199	210	223
Inoculation	40	-	0.4	0.45	-	0.15	-	0.35	0.27	0.15	0.23	-	-	-
Non Inoculation	40	0.15	0.19	0.07	0.49	0.10	0.10	-	0.05	-	0.11	0.37	0.34	0.12

It is important to know the composition of genotypes to study patterns of inheritance at the population level obtained from genetic contributions through marriage and adaptation processes and to avoid the important genotypes becoming extinct. The level of genetic diversity was indicated by the value of observed Heterozygosity (Ho), expected Heterozygosity (He) and Polymorphism Information Content (PIC). The highest and lowest Ho and PIC values (Table 4). PIC values at locus 6 PA 18 for the inoculated plant population showed a low value because the percentage of homozygous genotypes is more than heterozygote genotypes. However, the results of PIC calculations (0.534) indicated that the locus was still polymorphic and would be used for analysis. A locus was said to be polymorphic if the locus polymorphism was <0.95 and monomorphic if the value was> 0.95 [15].

Table 4 Analysis of per locus alleles for each population

Locus	Population	Number of Alleles	PIC	Ho	He
6 PA 18	Inoculation	3	0.534	0.3	0.631
	Non Inoculation	6	0.651	0.65	0.724
71 PA 17	Inoculation	4	0.679	0.45	0.747
	Non Inoculation	5	0.666	0.45	0.724

The average PIC value was quite high (0.642) indicating that *A. microcarpa* plant were more plants that reproduce through sexual reproduction by cross-pollination. The high level of heterozygosity was caused by the variety of sources of accession. In this study, the source of the plants came from the collection of *A. microcarpa* from various regions in the Carita Research Experimental Garden. Woody plants such as *A. microcarpa* which have a pollination system through crossing (out crossing) in general also have had a great variability [16]. Thus, heterozygosity in this study showed a high level.

The type of allele detected in 71PA17 was higher than the non inoculation population, but it did not affect the results of the calculation of heterozygosity because the calculation of heterozygosity involved the number of samples. Therefore, the average heterozygosity was not only influenced by the frequency of the detected alleles but also the number of samples observed. The average Ho was smaller than He in the Hardy-Weinberg equilibrium condition which meant there was an indication of a long-term trend of heterozygosity deficit in each population. Consequently, the genotype structure would lead to an increase in homozygotes

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[17]. Increased homozygotes in the long run will cause depression inbreeding that is not favorable for the development of *A. microcarpa* plant.

Genetic diversity

The value of genetic differentiation (Gst) would be shown through the average value of genetic diversity between populations (Dst), in populations (Hs) and the total value of genetic diversity (Ht). The small Gst value illustrates the high level of total population diversity because the higher the total population diversity the coefficient of genetic differentiation (Gst) is lower [15].

Table 5 Genetic diversity based on two microsatellite loci

Loci	n	p	A	Ap	He	Ho	Hs	Ht	Dst	Gst
6PA 18	80	1	6	6	0.78	0.617	0.706	0.778	0.071	0.092
71 Pa 17	80	1	7	7	0.82	0.517	0.723	0.822	0.099	0.121
Mean	80	1	6.5	6.5	0.80	0.567	0.714	0.800	0.085	0.107

Remarks: n = Number of samples

p = locus polymorphism rate (95% criterion- GDA)

A = Average number of perlokus alleles (Arlequin)

Ap = Number of private alleles (GDA)

He = Expected heterozygosity (Fstat)

Ho = Observed heterozygosity (Fstat)

Hs = Genetic diversity in populations

Ht = total genetic diversity

Dst = Distribution of genetic diversity between populations

Gst = Proportion of total diversity spread between populations

The value of genetic differentiation would be used to study genetic diversity both in population and between populations. The Hs value (0.723) at locus 71 PA17 was far greater than the DST value (0.121) which indicated genetic differences in the population were higher than between populations. Based on these data, it would be seen that the distribution of genetic diversity between populations was directly proportional to the proportion of total diversity that was spread between populations where the value of the locus 71 PA17 was higher compared to the 6 PA locus 18. The magnitude of the observed value indicated that variations occurred not only were due to the differences in genetic structure of each population but also were influenced by environmental factors. However, the high heterozygosity values of expectations indicated that genetic factors were relatively more involved than environmental factors. The value of heterozygosity was very useful as a parameter in the selection process. The traits used for selection should have heterozygosity values that were not always high, because they would be easily inherited.

Microsatellite Specific Alleles

The highest number of specific alleles were observed in non Inoculation and inoculated populations. The existence of these specific alleles would be caused by mutations in the microsatellite locus, which was estimated to occur at a rate of 10-5 to 10-4 per generation [18]. The detection of specific alleles, although of high value as specific markers of specific genotypes or characters, suggested the need for further evaluation of each individual plant of one variety to determine the possibility of heterogeneous varieties [19]. If heterogeneity was found between individuals in one variety, isolation was needed for several generations to increase the homogeneity of the variety concerned.

Table 6 Specific alleles of *A. microcarpa* at two microsatellite loci

The Locus	Allele	Frequency	Population
6 PA 18	180	0,15	Non Inoculation
6 PA 18	223	0,10	Non Inoculation
71 PA 17	210	0,3375	Non Inoculation
71 Pa17	223	0,125	Non Inoculation
71 Pa 17	178	0,150	Inoculation Inoculation

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71 PA 17	158	0,350	

Three alleles of the two loci (6 Pa18 and 71 Pa 17) were common alleles, and those were detected > 30% in all genotypes (Table 6). All microsatellite loci detected were rare alleles, with the frequency detected <5% of all genotypes analyzed. Intermediate alleles (5%≤frequency≤30%) and common alleles were account for 41% of all alleles obtained. Most rare alleles were also specific alleles, namely alleles that belong to a particular genotype.

These alleles would be a characteristic of a plant that would be the characteristic of a particular plant (fingerprinting) when it is associated with important useful genes [20]. Genotypes with alleles would rarely be a source of new genetic diversity for breeding and genetic improvement, especially if the alleles are related to important plant characteristics or resistance to biotic and abiotic stresses.

CONCLUSION

The results of this study indicated that *A. microcarpa* has a high level of genetic diversity. In several populations with testing of two amplified loci, specific alleles with low frequency were observed. These alleles show the uniqueness of the observed plants that would be used as a criterion for conservation. Thus, genetic diversity can be maintained. However, to get even better data, further research is needed to obtain certainty about these unique alleles, for further conservation action.

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